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(71) Applicant *(for all designated States except US)*: PIONEER HI-BRED INTERNATIONAL, INC. [US/US]; 800 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BIDNEY, Dennis, L. [US/US]; 8385 Plum Drive, Urbandale, IA 50322 (US). SCELONGE, Christopher, J. [US/US]; 5314 Ovid Avenue, Des Moines, IA 50310 (US). WANG, Lijuan [CN/US]; 4728 80th Place, Urbandale, IA 50322 (US).

(74) Agents: YATES, Michael, E. et al.; Darwin Building, 7100 N.W. 62nd Avenue, Johnston, IA 50131-1000 (US).

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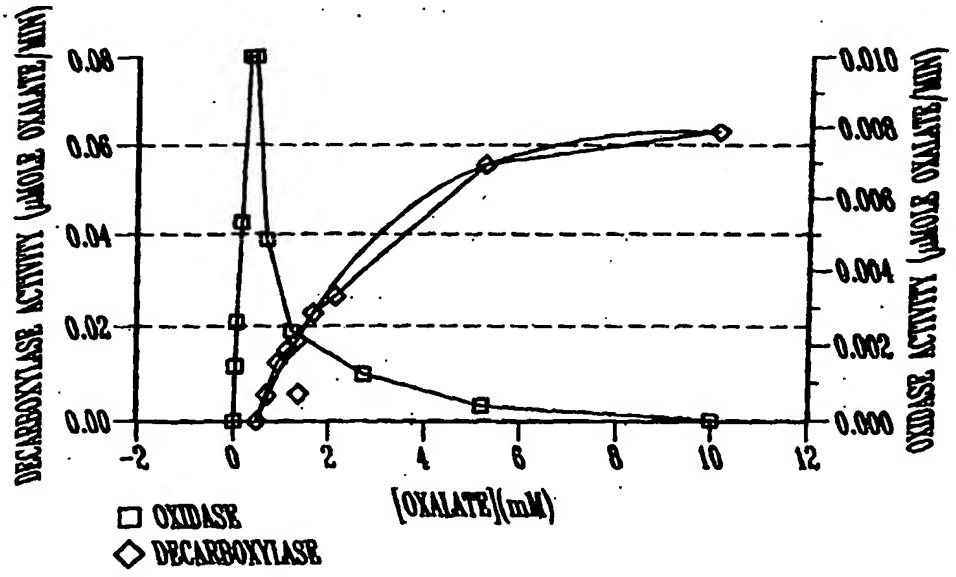

(57) Abstract

A method for the recovery of transformed plants without selectable markers, by the enrichment of transgenic sectors using nodal culture and non-selective screening assays, is provided. The enrichment is accomplished through non-selective assays of nodal explants and plant tissue developed therefrom. Plant tissue identified as positive for expression products of non-selectively assayable transgenes is recovered from culture for the production of fertile transgenic plants.

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RECOVERY OF TRANSFORMED PLANTS WITHOUT SELECTABLE MARKERS BY NODAL CULTURE AND ENRICHMENT OF TRANSGENIC SECTORS

FIELD OF THE INVENTION

The present invention relates to the recovery of genetically transformed plants from tissue culture. More particularly, the present invention relates to the recovery of transformed plants from culture, without the use of selectable markers, using nodal culture and enrichment of transgenic sectors.

BACKGROUND

The genetic transformation of plants for the introduction of foreign genetic material, or for the alteration of the native DNA of a plant, has in the last ten to fifteen years been the focus of a major research and development effort in the plant sciences. Millions of dollars and tens of thousands of hours of research have been spent in connection with the development of plant transformation techniques, the improvement of plant tissue culture methodologies, and the development and improvement of methods for the identification of successfully transformed plant cells and/or plants in tissue culture. While much progress has been made, in many cases the systems used today are still far from optimal, and it appears that research efforts in these areas will continue unabated for the foreseeable future.

In the area of agricultural genetics these efforts have been particularly significant, as have the developments in the field to date. Several methods have been developed for the insertion of foreign/exogenous DNA into the genome of plants (i.e., transformation methods), including, for example, microprojectile bombardment, electroporation, direct uptake, and insertion via *Agrobacterium tumefaciens*. See generally, Miki *et al.*, "Procedures for Introducing Foreign DNA into Plants", in *Methods in Plant Molecular Biology and Biotechnology*, Glick, B.R. and Thompson, J. E., Eds. (CRC Press, Inc., Boca Raton, 1993), pages 67-88. Also, expression vectors and *in vitro* culture methods for plant cell or tissue transformation and regeneration of plants are available. See, for example, McCormick *et al.*, Plant Cell Reports 5:81-84, 1986. In addition, a number of genes have been identified over the years as being useful in the process of selecting for successfully transformed plants from the tissue culture environment in which the genetic

transformations are typically carried out. In fact, it is safe to say that the use of selectable markers is now the standard method used to select for transformed plants in the commercial production of transgenic crops. Some examples of such selectable marker genes include the bar gene and the pat gene (which confer resistance to glufosinate herbicides) (see, *e.g.*, DeBlock *et al.*, EMBO J., 6:2513-2518, 1987), the NPTII gene (which confers resistance to the antibiotic kanamycin) (see, *e.g.*, Fraley *et al.*, CRC Critical Reviews in Plant Science 4:1-25, 1986), and the Hm1 gene (which confers resistance to the HC toxin of *Cochliobolus carbonum*) (see U.S. Patent Number 5,589,611). Many other selectable markers are known to those of skill in the art. See Bowen, B.A., "Markers for plant gene transfer", in *Transgenic Plants - Engineering and Utilization*, Vol. 1, Academic Press, N.Y., pp. 89-123.

Although there are a number of selectable marker genes from which to choose for selection of transformed plant cells or plants from tissue culture, and although the transformation methods currently practiced in the art are in some cases reasonably effective and efficient, there are still several problems with the current systems. For example, the wounding of sunflower meristems and the subsequent application of *Agrobacterium* has proven to be effective for sunflower transformation (European Patent 0 486 233B1; Bidney *et al.*, Plant Molec. Biol. 18:301-313, 1992). However, due to the nature of the transformation process, many of the resultant transgenic plants are chimeric for transgene expression. In other words, in many instances the transgenic plants that are regenerated from culture following the insertion of the foreign DNA may not be uniformly transformed; instead, only one quarter, or one half, or three quarters, or some fraction of the plant may be transformed. This is most likely due to the fact that only some of the meristematic cells are actually transformed by the *Agrobacterium*; those meristematic cells that are transformed give rise to transgenic sectors that develop into the transgenic portions of the chimeric plants.

Furthermore, this process is complicated, and in some sense impaired, by the use of selectable markers for the identification of successfully transformed plant cells, plant tissue, or whole plants. By its very nature the selection process, using selectable marker genes that are intended to impart resistance to a particular selective agent, involves the killing or retardation of growth of untransformed cells by the lethal, or at least strong, selective pressure applied by the use of the selective agent. In addition, however, such selective pressure can also, under certain circumstances, cause the loss of successfully transformed

cells or plants as well. For example, a successfully transformed cell might not be expressing the selectable marker gene product at a level sufficient to resist the selective agent; or the transformed cell might be at a developmental stage during which, in spite of the presence of and expression of the marker gene, the cell is particularly sensitive to the selective pressure. Or, in the case of a chimeric plant which is regenerating in culture, there may not be sufficient numbers of transformed cells in that particular chimeric plant for that plant to be able to resist the selective agent. In any case, the result is that the number of fully transformed plants recovered from the transformation and selection process is lower than might otherwise be expected. This has been seen to be the case in actual practice, as in, for example, the work of Ritala *et al.*, Plant Molec. Biol. 24: 317-325, (1994).

In addition, in many cases there are governmental regulatory concerns associated with the use of selectable marker genes in transgenic plants. This is particularly true when antibiotic resistance genes are used. It may also be true for the use of herbicide resistance genes in certain crop plant species that are closely related to wild species, for example sunflower and canola.

Therefore, there is a particular need in the field for alternatives to the selection process described above. A non-selective system for recovery of transformed plants would be particularly advantageous, especially if the system involved the use of genes which would foster recovery and enrichment of transformed plant cells and tissues which might otherwise be lost using a selectable marker system. Such a non-selective system could enhance the overall efficiency of the production of transgenic plants for research or commercial use. In addition, such a non-selective system would be advantageous if it were to use genes which would be less likely to have an environmental impact. Although use of non-selective markers for identification of transformed plants is known in the art, the present invention provides important advantages over known systems, in that in its preferred embodiments it uses an agronomically useful gene as the non-selective gene, and further, in all embodiments, it provides for enrichment of transgenic sectors in chimeric plants, allowing for the development of uniform, or at least near-uniform, transgenic plants. This is in contrast to, for example, the method of Christou *et al.*, as described in U.S. Patent No. 5,015,580, which relies on lethal selection, or screening using either the β-glucuronidase (GUS) or the firefly luciferase gene. It is important to note specifically that the method of Christou *et al.*, typically results in chimeric plants, even when screening is

used, as discussed, for example, in Example 8, at column 20, line 4 of the Christou *et al.* patent.

SUMMARY

The present invention addresses the need for a non-selective system for regeneration of transformed plants from culture by providing a method for recovery of transgenic progeny using nodal culture and non-selective enrichment of transgenic sectors. For example, it is known that wounded, *Agrobacterium*-treated sunflower meristems produce sectors of plant tissue which express transgenes (Bidney *et al.*, Plant Molec. Biol. 18:301-313, 1992). The present invention takes advantage of the finding that nodal cultures from such regions of parental, T0 shoots can produce plants that are enriched in what started out as a limited region of transgene expression. Stem segments with associated nodes from apparent regions of transformation are re-cultured to induce nodal meristem development. Such shoots can be further enriched and recovered to the greenhouse, and can produce progeny. Nodal recovery and non-selective enrichment, following the use of appropriate transformation methods for transgene introduction, can allow recovery of transgenic progeny without the use of a selectable marker.

The present invention provides a method for recovering transformed plants from culture. Once plant cells or tissues have been transformed with foreign/exogenous DNA comprising a non-selective assayed transgenic using any appropriate art-recognized method, the first step of the method of the invention is to culture the transformed plant cells or tissue until nodes comprising meristematic tissue have developed. At this stage the plant tissue is assayed using the appropriate non-selective assay. Nodal explants are then prepared from the assay-positive tissue. The nodal explants are then cultured so that shoots form from the explants. The shoots are then cultured to provide for further shoot and leaf formation. The non-selective assay is then repeated, and assay-positive shoots are recovered. T0 plants, with enriched transformed sectors, are then recovered from the assay-positive shoots.

In further embodiments of the present invention, the method is extended by performing further assays on the T0 plants. Non-transformed (assay-negative) sectors are removed, and shoots are recovered from assay-positive transgenic sectors. Uniform, or nearly uniform chimeric transformed T0 plants are then produced from the recovered shoots.

In an additional further embodiment, seed is produced from the transformed T0 plants, and transformed T1 plants are germinated from those seeds. Extending the invention further, seeds are produced from the transformed T1 plants, and transformed T2 plants are germinated therefrom.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the oxalate dependence on rate for *Aspergillus* decarboxylase (pH 5.0) and barley oxidase (pH 3.5).

FIGURE 2 shows a re-plotting of the data shown in FIGURE 1.

FIGURE 3 shows the pH range of oxalate oxidase and oxalate decarboxylase.

FIGURE 4 shows an oxalate oxidase assay performed on pooled meristem explant leaf tip from *in vitro* plantlets of *Agrobacterium* non-selection experiments.

FIGURE 5 shows a plasmid map of plasmid PHP9755.

FIGURE 6 shows a plasmid map of plasmid PHP10521.

FIGURE 7 shows a plasmid map of plasmid PHP10092.

DETAILED DESCRIPTION

Nodal culture and enrichment of transgenic sectors of chimeric transformed plant tissue, using non-selective assays which allow for the recovery of transformed plants without the use of selectable markers, are provided. Following the transformation of plant cells in cell or tissue culture, using transformation methods recognized in the art as useful for the insertion of one or more transgene(s) into plants, an assay for transformed cells or tissue using a non-selective assay process is performed. Once this initial screen for positive transformants has been performed, assay-positive nodal explants containing meristematic tissue are prepared. The nodal explants are allowed to develop in culture, and later in the development process plant tissue, typically shoot or leaf tissue, is subjected to further non-selective assays in a process for enrichment of transgenic sectors. The enriched sectors are further enriched to obtain uniform, or near-uniform, transgenic plants.

As used herein, the terms "meristem", "axillary bud", and "adventitious bud" refer to plant structures as defined by K. Esau in *Plant Anatomy*, John Wiley & Sons, Inc., New York, 2nd Ed., 1965. At page 67 Esau defines "meristem" as "perpetually young tissues, primarily concerned with the formation of new cells." Esau notes, at page 70, that the most common grouping of meristems is "based on their position in the plant body", and

that this classification "divides the formative tissues in *apical meristems*, that is, meristems located at the apices of main and lateral shoots and roots, and *lateral meristems*, that is, meristems arranged parallel with the sides of the organ in which they occur." With regard to "axillary buds", Esau states, at pages 109-111, that in seed plants "branches commonly are formed in close association with the leaves and in their nascent state are referred to as *axillary buds*. If the axillary bud develops into a shoot, its apical meristem is gradually organized - commonly duplicating the pattern found in the parent shoot apex - and proceeds with the formation of leaves." Esau defines adventitious buds as "buds that arise without connection with the apical meristem from more or less mature tissues.... Adventitious buds occur on stems, roots, and leaves on intact plants and on isolated cuttings or leaves. In cuttings the buds usually are initiated in callus tissue, which develops before the buds." Esau at page 112.

In addition, as used herein the term "nodal meristem" refers to meristematic tissue located at a nodal region of a plantlet which will give rise to an axillary bud or an adventitious bud, and which in turn can give rise to a functional shoot. "Nodal explants" are subdivisions of plantlets having expanded internodes, the subdivisions having been made by physically dividing the plantlet into "nodal segments." See generally, Grout, B.W.W., "Meristem-Tip Culture", in *Methods in Molecular Biology*, vol. 6, *Plant Cell and Tissue Culture*, Pollard and Walker, Eds., The Humana Press, Clifton, N.J., 1990 (Chapter 9, page 82). Nodal explants can be prepared from shoots generated from embryonic axis meristem, lateral or axillary meristem, or adventitious meristem. Thus, the nodal explants will comprise nodal meristematic tissue.

As noted previously, several transformation methods are recognized to be useful for the introduction of transgenes into plants. See, *e.g.*, Miki *et al.*, "Procedures for introducing foreign DNA into plants", in *Methods in Plant Molecular Biology and Biotechnology*, Glick, B.R. and Thompson, J. E., Eds. (CRC Press, Inc., Boca Raton, 1993), pages 67-88. The particular method selected will vary depending upon the type of plant to be transformed, the experience of the practitioner, and other factors. The transformation method used to prepare the transformed plants to be recovered and enriched using the present invention is not critical to the present invention, since the present invention relates to a method for recovery of transformed plants post-DNA insertion.

The present method involves subjecting transformed plant cells/tissues, in culture, to non-selective assays that can determine the presence and/or expression of transgenes. The results of the assay allow for the identification and enrichment of transgenic sectors in a process that ultimately yields near-uniform (chimeric), or wholly uniform, transgenic plants. As used herein, "transgene" means a gene or polynucleotide that has been inserted into a plant using a transformation method. The transgene may be "foreign" in the sense that it was isolated for insertion into the plant from an entirely different organism (meaning any organism not of the same species as the plant being transformed); or the transgene may be "exogenous" in the sense that it is a synthetic gene or an additional copy of a gene that is already present in the plant being transformed.

The present invention envisions the use of several types of "non-selectively assayable transgenes" in the practice of the invention. In one embodiment the non-selectively assayable transgene is one which produces, upon expression, a product which can be assayed for using non-lethal methods. This will typically mean that there is some sort of chemical, biological, or physical assay available which will determine the presence or absence or change in amount of the expression product of the gene. In certain embodiments in which the non-selectively assayable transgene produces an enzyme involved in a metabolic pathway, the assay may determine the presence or absence of, or a change in the amount of, a metabolite produced directly by the enzyme, or the presence or absence of, or a change in the amount of the final product of the metabolic pathway, rather than the presence or absence of the expression product (the enzyme) itself. For example, such an enzyme might be involved in a metabolic pathway which produces oils having a particular fatty acid makeup. It will also be apparent to those of skill in the art that many forms of assay techniques will be available for the practice of the invention. For example, any expressed protein capable of detection by ELISA could be assayed by using the associated ELISA; or a modification in the amount of a specific fatty acid could be determined using the appropriate biochemical analytical technology (GCMS, for example); or a bioassay could be used (for example, expression of a crystal protein toxin from *Bacillus thuringiensis* (Bt) could be determined by screening for deleterious effects of transformed plant tissue on insects or insect larvae that are susceptible to the crystal protein toxin).

Those of skill in the art will also recognize that the presence of the non-selectively assayable transgene can be detected directly using DNA amplification techniques known in

the art, including, but not limited to PCR, RT-PCR, or LCR, for example. By way of illustration, the non-selectively assayable transgene could be an embryo-specific gene such as a desaturase under the control of an embryo-specific promoter. Genetic modification using such a gene construct would be expected to modify seed oil profiles, without affecting expression in leaves. A properly performed PCR screen would detect the presence of the sequence in transformed plants, and the inventors have successfully screened for transgenics in T1 seed populations by PCR after transformant recovery. Those of skill in the art will recognize that when *Agrobacterium*-mediated transformation has been used as the transformation method, certain measures will be necessary to distinguish the non-selectively assayable transgene present in *Agrobacterium* from the transgene present in the genome of transformed plant cells. The use of an appropriate set of primers that includes primers for *Agrobacterium* DNA will address this issue. It will also be recognized that any gene that can be amplified using amplification technology such as PCR can serve as a non-selectively assayable gene in this embodiment of the invention.

Several candidate non-selectively assayable transgenes genes that can be inserted into plant cells or tissues can, when expressed, be assayed for in the non-selective process of the invention. In preferred embodiments of the invention the assayable gene will also constitute a gene that confers an agronomic trait of value to the plant. Examples of non-selectively assayable transgenes include, but are not limited to, the oxalate oxidase gene which has been isolated from wheat (Dratewka-Kos *et al.*, J. Biol. Chem. 264:4896-4900 (1989)) (the "germin" gene) and barley (WO 92/14824), the oxalate decarboxylase gene (which has been isolated from *Aspergillus* and *Collybia* [see WO 94/12622]), other enzymes that utilize oxalate, the gene encoding the Green Fluorescent Protein (GFP) and variants thereof, luciferase genes, anthocyanin markers, other oxidase enzymes such as polyphenol oxidase, glucose oxidase, monoamine oxidase, choline oxidase, galactose oxidase, l-aspartate oxidase, and xanthine oxidase, and the like. As those of skill in the art will recognize, the assay for the gene expression product will vary with the nature of the expression product; for example, an enzymatic assay can be used in those instances where the expression product is an enzyme, such as in the case of transformation with a gene encoding oxalate oxidase or oxalate decarboxylase. A visual or colorimetric assay would be appropriate for plant cells or tissues transformed with a GFP gene. As those skilled in the art will also recognize, when an enzymatic assay is appropriate the existence of an assay in the art would be particularly useful. Furthermore, as noted above, other assay

techniques (e.g., PCR for the non-selectively assayable transgene itself, or ELISA, or a bioassay, or chemical analytical methods such as GCMS) will be appropriate in the performance of the various embodiments of the invention.

In a further alternative embodiment of the present invention, the non-selective assay can involve a procedure which measures a loss of, or a decrease in the level of expression of, a measurable product which is normally present or which is normally expressed at higher levels. For example, antisense or co-suppression technology can be used to down-regulate the expression of a particular gene, and an appropriate assay which would detect the disappearance of or decrease in amount of the expression product or a metabolic product can be used.

The present invention, in its preferred embodiments, utilizes repeated application of the appropriate non-selective assay at various stages of the development of the plants in culture. Initially, the assay is performed within a relatively short time after transformation. Those of skill in the art will recognize that this time will vary depending upon the plant species that has been transformed; however, the period will be at least as long as necessary for the development of shoots that are large enough for physical isolation of nodal explants. The nodal explants are prepared from tissues that provide the appropriate "assay-positive" results upon performance of the non-selective assay used (in this specification the term "assay-positive" refers to non-selective assay results that indicate to the practitioner that the non-selectively assayable transgene has been stably inserted into the plant cells). For example, nodal explants would be prepared from tissue that assayed positive for the transgene (using PCR) or for the transgene product (using, for example, an oxalate oxidase enzyme assay). These nodal explants are then cultured so that shoots form from each node. Various aspects of the use of nodal explants in culture and the subsequent development of shoots, and the micropropagation of plants therefrom, are discussed generally in Grout, B.W.W., "Meristem-Tip Culture", and in Evans, N.E., "Micropropagation: Axillary bud manipulation", in *Methods in Molecular Biology*, vol. 6, *Plant Cell and Tissue Culture*, Pollard and Walker, Eds., The Humana Press, Clifton, N.J., 1990 (Chapters 9 and 10, pages 81-91 and 93-103, respectively), as well as in Werbrouck and Debergh, "Applied aspects of plant regeneration: 6A. Micropropagation", in *Plant Cell Culture: A Practical Approach*, Dixon and Gonzales, Eds., IRL Press, New York, 1994, pages 127-135. See also Nauerby *et al.*, "A rapid and efficient regeneration system for pea (*Pisum sativum*), suitable for transformation", Plant Cell Reports 9:676-679, 1991;

Patnaik and Debata, "Micropropagation of *Hemidesmus indicus* (L.) R. Br. through axillary bud culture", Plant Cell Reports 15:427-430, 1996; Demeke and Hughes, "Micropropagation of *Phytolacca dodecandra* through shoot-tip and nodal cultures", Plant Cell Reports 9:390-392, 1990; Alexandrova *et al.*, "In vitro development of inflorescences from switchgrass nodal segments", Crop Sci. 36:175-178, 1996; and Pieper and Smith, "A whole plant microculture selection system for kentucky bluegrass", Crop Sci. 28:611-614, 1988. Chowrira *et al.* ("Electroporation-mediated gene transfer into intact nodal meristems *In Planta*", Molecular Biotechnology 3:17-23, 1995) report the electroporation-mediated transfer of genes into intact nodal meristems *in planta*, but the methodology described therein differs significantly from the present invention, since the techniques of Chowrira *et al.* specifically relate to a transformation method for generating transgenic plants without *in vitro* tissue culture.

Shoots that develop from the nodal explants are then transferred to an appropriate medium that will allow continued shoot development. Following this period of continued shoot development and leaf formation the appropriate non-selective assay is repeated. At this time, assay-positive shoots recovered from a single node will generally have been enriched in the transgenic sector detected in the initial assay (the assay performed prior to nodal culture).

Following the identification of the enriched transgenic shoots, the necessary manipulations for further development of T0 plants are carried out. For example, but not by way of limitation, enriched transgenic sunflower shoots can be rooted, or grafted to *in vitro*-grown sunflower seedling rootstock. For other species those of skill in the art will recognize that shoots recovered from culture can, in general, be rooted *in vitro*, without grafting, using standard techniques well known in the art. Those of skill in the art will also recognize that the specific techniques used for this manipulation will vary depending upon the plant species being recovered.

In the practice of the invention, transformed sectors of the T0 plants are then identified by another repetition of the appropriate assay. Following the assay, non-transformed sectors are trimmed off and axillary buds from the transgenic sectors are recovered so as to obtain uniform or near-uniform transformation events. Selfed seed, or outcrossed seed (or any seed from a T0 plant pollinated by another plant source) from these T0 plants is then collected, germinated, assayed again, and selfed. Seed from

confirmed T1 transgenic plants (seed which will produce T2 plants) are then used in field trials.

As will be recognized by those of skill in the art, the present invention is useful for the recovery of transformed plants of a variety of species. Indeed, the present method can be practiced for recovery of transgenic plants of any species amenable to nodal culture. Such species include, but are not limited to, soybean, sunflower, corn, wheat, rice, barley, canola, alfalfa, and vegetable species.

In the development of the present invention, chimeric sunflower plantlets expressing GUS gene were studied and the optimum condition for production of early sectored transgenic events was determined. See Bidney *et al.*, Plant Molec. Biol. 18:301-313, 1992. Following those protocols, but using a GFP gene, heritable GFP events were successfully obtained in a non-selection protocol. Transgenic events expressing GFP were identified by the detection of green fluorescence of small shoots *in vitro*, followed by the removal of non-fluorescing shoots and sectors. Stem nodes from GFP expressing sectors were recovered, shoot development promoted, and transgenic sectors were mapped in greenhouse-established plants, from which progeny were recovered. Desirable transformation efficiency was achieved. Similar results have been achieved using oxalate oxidase, oxalate decarboxylase, and crystal protein genes from *Bacillus thuringiensis* (Bt), as discussed in the examples set forth below.

Having generally described the invention, the following examples are offered by way of illustration and not by way of limitation.

Example 1

This Example describes the results of oxalate oxidase and oxalate decarboxylase enzyme assays. These studies were performed using commercially available oxidase and decarboxylase enzymes. The studies were performed prior to obtaining transgenic plants expressing either enzyme, and were performed to gain some insight concerning the kinetic properties of the two oxalate degrading activities. The enzymes used were barley oxidase (pH 3.5 optimum) (Boehringer) and *Aspergillus* decarboxylase (pH 5 optimum) (Boehringer). The information obtained was used in the adaptation of assays for analysis of transgenic material.

Enzyme assays were conducted at concentrations believed to approximate the "transgenic level of expression." Sunflower leaf 35S::GUS expression was calculated at 2.3μg/ml, 3.3μg/ml for barley oxalate oxidase and at 6.47μg/ml for decarboxylase. The

Oxalate dependence on rate for decarboxylase (pH 5.0) is plotted in Figure 1 with barley oxidase (pH 3.5) data. Decarboxylase is not inhibited by increasing levels of oxalate and the Km of the reaction is approximately 2×10^{-3}M (1.2×10^{-4}M for barley oxalate oxidase). Note that the scales of the double Y plot in Figure 1 are different; re-plotting the data on the same scale generates the curves shown in Figure 2.

The pH range of oxalate oxidase and oxalate decarboxylase was determined, and the data plots are shown in Figure 3.

Direct comparison of the two enzymes is difficult in that decarboxylase is inactive at the oxidase pH3.5 optimum, while oxidase has lost 80% of its pH 3.5 activity at the decarboxylase pH optimum of 5.0. It is clear, however, in assays run at the appropriate pH, that oxalate oxidase is maximally active at low oxalate concentrations, while decarboxylase activity is below the limits of detection. Decarboxylase is very active at oxalate levels that significantly inhibit oxidase activity. Calculations based on the initial rates of reaction from the linear portions of the velocity versus substrate curves show that oxalate decarboxylase is about one-half as active as barley oxalate oxidase based on μmoles of oxalic acid converted per minute. As those of skill in the art will appreciate, these factors will be appropriate for consideration in choosing a non-selective gene and assay for the practice of embodiments of the present invention involving the use of such enzymatic assays.

Example 2

This Example describes an assay for oxalate oxidase that can be used in the practice of the present invention. Oxalate oxidase converts oxalate to CO_2 and H_2O_2. The gene for this enzyme has been cloned from wheat (Dratewka-Kos *et al.*, J. Biol. Chem. 264:4896-4900 (1989)), and this gene was used in the generation of transgenic plants (see Example 4 below).

Expression of oxalate oxidase in transformed plant cells and tissues can be detected by the enzyme assay described by Sugiura *et al.*, Chem. Pharm. Bull. 27 (9) 2003-2007 (1979). The assay was adapted to megatiter plates so that a large number of samples could be screened. Leaf tissue samples were collected and placed in megatiter tubes and homogenized in 400μl of 0.1 M Na-succinate buffer (pH 3.5). Supernatants were decanted and pellets were resuspended in 400 ul of the succinate buffer (pH 3.5). Next, 50μl of 10mM oxalate (pH 3.5) was added to each tube. The tubes were incubated at room temperature for 3-4 hours, with shaking. After centrifugation at 4000 rpm for 20

minutes, 100 µl of the supernatant, 17.5 µl of 1M Tris free base solution, and 82.5 µl of developing solution (8 mg of 4-aminoantipyrine, 400 µl of peroxidase, and 20 µl of N, N-dimethylaniline dissolved in 100 ml of 0.2 M Tris-HCl, pH 7.0) were transferred into a microplate (see Figure 4). The absorbances of the resulting color in the microplate wells were measured at 550 nm.

An alternative oxalate oxidase assay useful in the practice of the present invention is as follows: Leaf tissue was lyophilized and ground to a fine powder. The powder was resuspended in sodium succinate buffer (0.1M, pH 3.5) plus a drop of 1mg/ml Tween-20. Individual 1ml reaction tubes were set up by adding 100µl of 10mM oxalate in 0.1M sodium succinate buffer (pH 3.5), and 800µl of succinate buffer, and 100µl of the resuspended leaf extract. The tissue extract was added last to start the reaction timing. The reaction was allowed to proceed for a defined time (1-30 minutes) with agitation, and 100µl of the reaction mix was transferred to microtitre plate wells containing 17.5ul of 1M Tris free base. Next, 82.5µl of peroxidase-linked color development solution (8mg 4-aminoantipyrine, 20µl N,N-dimethylaniline, 400µl peroxidase, all in 100ml of 0.2M Tris-HCl, pH7.0) was added, and absorbance was read at 550nm. For time course assays successive 100ul aliquots are removed at desired times. Values based on initial dry weights can be used to compare different samples and/or plants.

Example 3

This Example describes an assay for oxalate decarboxylase that can be used in the practice of the present invention.

Expression of *Flammulina* oxalate decarboxylase in transformed plant cells and tissues can be detected by the enzyme assay described by Labrou *et al.*, "Biomimetic dye-liquids for oxalate-recognizing enzymes studies with oxalate oxidase and oxalate decarboxylase." J. Biotech. 40:59-70, 1995. Decarboxylase activity is linked to a second activity, that of formate dehydrogenase, that will oxidize the decarboxylase-generated formate, with the subsequent reduction of NAD to NADH (Johnson *et al.*, Biochem. Biophys. Acta 89:351, 1964). The increase of OD340 as NAD is reduced is used to generate an initial reaction rate that is linear with respect to formate concentration from 0.2 to 2.0 µmole. The assay was adapted to megatiter plates (Continental Lab Products, San Diego, CA) so that a large number of samples could be screened. Leaf tissue samples were prepared as in Example 2. After centrifugation at 4000 rpm for 20 minutes (as in Example

2), 100µl of each supernatant were transferred to microtiter plate wells and 17.5 µl of 1M Tris free base solution was added to each. Next, 10µl of B-NAD (6.6mg/ml stock, Sigma) were added to each sample well. The samples were mixed, and 5µl formate dehydrogenase (4.0 mg/ml stock, 1 enzyme unit/mg solid, Sigma) was added. The absorbance at 340nm was measured repeatedly over a 10 minute period to generate a reaction rate curve, and the slope of the initial rate was determined.

Example 4

Non-selection protocols have been conducted using the enzyme assays of oxalate oxidase and oxalate decarboxylase as screenable markers to identify transgenic sectors in T0 plantlets. The protocols of the oxalate oxidase assays are described in this example. The decarboxylase methods were the same, aside from the use of the decarboxylase assay, described in Example 3 herein, for screening.

Plant Materials: Mature seeds of Pioneer Sunflower line SMF-3 were used for the transformation. Mature seeds of Pioneer hybrid sunflower line 6440 were used as a rootstock for the grafting of transformed shoots.

Agrobacterium Strain and Plasmid: The infection of the meristems was carried out (following wounding, see "Transformation", below) using *Agrobacterium tumefaciens* strain EHA105 containing PHP9755 (Figure 5), a binary plasmid that contains a germin gene for the degradation of oxalic acid ($HOOC\text{-}COOH + O_2 \rightarrow 2CO_2 + H_2O_2$). The germin/oxalate oxidase gene product was detected using the oxalate oxidase assay described in Example 2. PHP9755 is an *Agrobacterium* binary plasmid that contains the Super MAS promoter driving expression of the oxalate oxidase gene and pinII terminator. The super MAS::oxalate oxidase cassette was first assembled in a pUC plasmid backbone by ligating an *Nco*I site flanking the 3' end of the Super MAS promoter to an *Nco*I site overlapping the start codon of oxalate oxidase. The pinII terminator was blunt end ligated 21 bases downstream of the stop codon of oxalate oxidase. The cassette, flanked by *Sal*I and *Eco*RI sites, was inserted between the T-DNA borders of a pBIN19 plasmid backbone. The Super MAS promoter was oriented proximal to the left T-DNA border. Alternatively, binary plasmid PHP10521 (Figure 6) was used in experiments in which the *Flammulina* oxalate decarboxylase (FVOXD) gene product was detected using the assay described in Example 3. PHP10521 is an *Agrobacterium* binary plasmid that carries a plant expression cassette consisting of the Super MAS promoter (Gelvin), germin signal sequence (Lane, G., Bernier, F., Dratewka-Kos, E., Shafai, R., Kennedy, T., Pyne, C., Munro, R., Vaughn,

T., Walters, D., Altomare, F., 1991. "Homologies between members of the germin gene family in hexploid wheat and similarities between these wheat germins and certain *Physarum* spherulins." J. Biol. Chem. 266, 10461-10469), FVOXD coding sequence, and pinII termination signal (An, G., Mitra, A., Choi, H., Costa, M., An, K., Thornburg, R., Ryan, C., 1989, "Functional analysis of the 3' control region of the potato wound-inducible proteinase inhibitor II gene." Plant Cell 1, 115-122). The coding sequence was constructed by synthesizing the germin signal sequence and fusing it to a DNA encoding the amino terminus of the mature FVOXD sequence. The deduced amino acid sequence of FVOXD had an amino terminus which was presumed to be a signal peptide. A strategy was designed to remove the signal, leaving the mature FVOXD protein, and replace it with the germin signal sequence. Two PCR clones were generated, one representing the mature FVOXD protein with an added *Nco*I restriction site at the 5' end and a second consisting of the germin signal sequence with a *Bam*HI site at the 5' end and an *Nco*I site at the 3' end. Two oligomers (PHN11260 5'GATCCATGGGTTACTCAAAGACCTTGGTTGCTGGTTTGTTCGCTATGTTGTTG TTGGCTCCAGCTGTTTTGGCTAC3' (SEQ ID NO: 1) and PHN11265 5'CATGGTAGCCAAAACAGCTGGAGCCAACAACAACATAGCGAACAAACCAGC AACCAAGGTCTTTGAGTAACCCATG3' (SEQ ID NO: 2)) were annealed to create a double stranded DNA fragment representing the germin PCR clone, the entire signal region and the initiating methionine, with the newly added restriction sites at the termini. The FVOXD PCR clone was generated by designing oligomers which annealed at the beginning of the mature protein sequence (FVOXD aa #26) and at a proximal downstream region with useful restriction sites. The 5' primer (PHN11380 5'GGTCCATGGTGCCTTTGGCGTCCACCAC3') (SEQ ID NO: 3) includes an *Nco*I site added to the 5' terminus. The 3' primer PHN11271 (5'TGCCGCCGAGCCCAGCCAC3') (SEQ ID NO: 4) spans a *Pst*I site near FVOXD aa #124. The native FVOXD gene was digested with *Pst*I to remove the 5' end of the gene. Then the 300 base pair purified, digested FVOXD PCR fragment and the smaller germin PCR clone were ligated to the remainder of the native sequence creating the modified gene with a 5' *Bam*HI site. The germin signal sequence/FVOXD encoding sequence fusion was then inserted downstream of the Super MAS promoter at *Bam*HI, and upstream of the pinII terminator by blunt ligation, to create the Super MAS::germin signal sequence/FVOXD encoding sequence::pinII cassette. PHP10521 was constructed by inserting the FVOXD encoding

cassette into the TDNA region of a pBin19 plasmid backbone (Bevan, M., 1984, Binary Agrobacterium vectors for plant transformation. Nucl. Acids Res 12, 8711-8721) with the 5' end of the Super MAS promoter proximal to the left TDNA border. The NOS-NPTII-NOS cassette between the T-DNA borders of Bin19 was removed.

Explant Preparation: Seeds were dehulled and surface-sterilized for 20 minutes in a 20% Chlorox™ bleach solution with the addition of two to three drops of Tween 20 per 100 ml of solution, and then rinsed three times with distilled water. Sterilized seeds were imbibed in the dark at 26 C for 20 hours on filter paper moistened with water. The cotyledons and root radical were removed, and the meristems explanted. The meristem explants were cultured on 374E (gibberellic acid (GBA) medium consisting of MS salts (see, generally, Skirvin, R.M., In: *Cloning Agricultural Plants Via In Vitro Techniques*, B.V. Conger, Ed., CRC Press, Knoxville, Tenn., pp. 51-140, 1981) Shepard vitamins (*Id.*), 40 mg/l adenine sulfate, 3% sucrose, 0.5 mg/l 6-benzylaminopurine (BAP), 0.25 mg/l indole-3-acetic acid (IAA), 0.1mg/l gibberellic acid (GA3), and 0.8% Phytagar™ (Gibco BRL), at pH 5.6, for 24 hours in the dark. The primary leaves were removed to expose the apical meristem. Approximately 40 explants were placed with the apical dome facing upward in a 2 cm circle in the center of 374M (GBA medium as described, except with 1.2% Phytagar), and then cultured on the 374M medium for 24 hours in the dark.

Transformation: (The transformation protocol used in this Example is that described in Bidney *et al.*, Plant Molec. Biol. 18:301-313, 1992). Approximately 18.8 mg of 1.8μm tungsten particles were suspended in 150 μl absolute ethanol, and sonicated for 2-4 seconds. After sonication, 8 μl of the suspension was dropped on the center of the surface of a macrocarrier. Each plate of meristem explants was bombarded twice with 650 psi rupture discs in the first shelf at 26 mm of Hg helium gun vacuum, using a BioRad helium gun.

Plasmid PHP9755 was introduced into *Agrobacterium* strain EHA 105 (see above) via freeze-thawing as described by Holsters *et al.*, Mol Gen Genet. 163:181-187 (1978). *Agrobacteria* were grown overnight at 28 C in a liquid "YEP" medium (10g/l yeast extract, 10g/l Bactopeptone, and 5g/l NaCl, pH7.0) in the presence of kanamycin. A pellet of this *Agrobacterium* culture was suspended in an inoculation medium (12.5mM 2-(N-morpholino) ethanesulfonic acid, 1 g/l NH4Cl, and 0.3 g/l MgSO4, at pH 5.7), to a final calculated concentration of *Agrobacteria* of 4.0 at OD 600. Particle-bombarded explants were transferred to 374E medium, and a droplet of the *Agrobacteria* suspension was

placed directly onto the top of the meristems. The explants were co-cultivated on the medium for 4 days, after which the explants were transferred to 374 C medium (GBA with 1 % sucrose and with no BAP, IAA, or GA3, and supplemented with 250 µg/ml cefotaxime). The explants were cultured on this medium for about 2 weeks under 16 hours of daylight, at 26 C.

Recovering Nodes and Plants: Following the two weeks of culture in 374C medium, the explants (approximately 2cm long after two weeks) were screened using the oxalate oxidase assay described in Example 2. Leaf samples of all plantlets from an explant were collected and pooled into a megatiter tube. After oxalate oxidase positive explants were identified, those shoots that failed to exhibit oxalate oxidase activity were discarded, and all positive explants were sliced into nodal explants, with each sliced piece containing at least one potential node. Small plantlets were sterilely sectioned by cutting the stem into segments at approximately 1mm above and below all identifiable nodal junctions with a number 15 scalpel blade. These nodal explants, or nodal segments, were then cultured on 374E medium for three to four days to induce nodal meristem and shoot development. Developing shoots were separated, and the nodal explants were then transferred to 374C medium for an additional four week culture period to promote shoot/plantlet development. Following the four weeks of culture on 374C medium, leaf samples from each recovered shoot were again screened using the oxalate oxidase assay. At this time the enzyme positive shoots that were recovered from single nodes were generally found to have been enriched in the transgenic sector detected by the initial oxidase assay that was performed prior to nodal culture.

Recovered oxidase-positive shoots were grafted to any of Pioneer sunflower hybrids 6440, 6150, 6351, 6338, 6464, or 6482 *in vitro*-grown sunflower seedling rootstock. The seeds were dehulled and surface-sterilized for 20 minutes in a 20% Chlorox™ bleach solution with two to three drops of Tween20 per 100 ml total volume, and were rinsed three times with distilled water. The sterilized seeds were germinated for three days on filter paper moistened with water, then transferred into "48 Medium" (one-half strength MS salts, 0.5% sucrose, 0.3% gelrite, at pH 5.0) and grown at 26 C in the dark for 3 days, then incubated at 16 hour day culture conditions. The upper portions of selected seedlings were removed, a vertical slice was made in each hypocotyl, and a transformed shoot was inserted into the vertical slice. The cut area was wrapped with parafilm, and after one week culture on the medium, the grafted plants were transferred to

soil. In the first two weeks they were maintained under high humidity conditions to acclimatize to the greenhouse environment.

Transformed sectors of T0 plants were identified by additional oxalate oxidase assays of the *in vitro* positive grafted shoots. After assay, non-transformed sectors were trimmed off and auxiliary buds from transgenic sectors were recovered so as to obtain uniform or near uniform transformation events. Seed from T0 plants were collected and germinated. The resultant T1 plants were then characterized for oxidase activity, and the transgenic plants were selfed. Seed from confirmed T1 transgenics (seed which would give rise to T2 plants) will be used in field evaluations. Good efficiency was achieved at the T0 stage: Sixty-seven shoots were recovered from 1280 explants, and 28 shoots were oxalate oxidase positive, for an efficiency of 2.2%. Transgenic progeny have been recovered from these plants, and 60-70% of transgenic T0 events are generally recovered to progeny.

Example 5

Non-selection assays have been conducted using an ELISA specific for the Bt crystal protein toxin Cry1F. Methods for the development of ELISA protocols for protein products are well known in the art. See, for example, Immunochemistry of Solid-Phase Immunoassay, J.E. Butler, Ed., CRC Press, Boca Raton, 1991.

The *Agrobacterium* strain EHA 105 (see Example 4, above) was used. The binary vector PHP10092 (Figure 7) was constructed using methodology similar to that in Example 4, but contained only the Cry1F gene under the direction of the Super MAS promoter. Plant material, explant preparation, Agrobacterium and plant transformation, recovery of nodal explants, shoots, and plants, and transgenic characterization were performed as described in Example 4, except that oxalate oxidase enzyme screening assays were replaced with Cry1F ELISA protocols. The data presented in Table 1 demonstrates that ELISA detection methods are useful in the practice of the present invention for identifying transgenic sectors in shoots.

TABLE 1

Category	Number
Total Explants	342
Explants with Cry1F ELISA-Positive Pooled Leaf Samples	26
Independent Cry1F ELISA-Positive Plantlets Recovered from Nodal Explants	5

Although the foregoing invention has been described in some detail by way of illustration and example, for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method for recovering transformed plants from culture, the method comprising culturing transformed plant cells or tissue comprising a non-selectively assayable transgene until nodes comprising meristematic tissue have developed; assaying the plant tissue using a non-selective assay; preparing nodal explants from the assay-positive tissue; culturing the nodal explants so that shoots form; culturing the shoots so that additional shoots and leaves form; and producing T0 plants with enriched transformed sectors from the recovered shoots.
2. The method of claim 1 further comprising repeating the non-selective assay on the additional shoots, and recovering assay-positive additional shoots.
3. The method of claim 1, further comprising assaying tissue of the T0 plants; removing non-transformed sectors; recovering shoots from assay-positive transgenic sectors; and producing uniform or chimeric transformed T0 plants therefrom.
4. The method of claim 3, further comprising producing seed from the transformed T0 plants, and germinating transformed T1 plants therefrom.
5. The method of claim 4, further comprising producing seed from the transformed T1 plants, and germinating transformed T2 plants transformed therefrom.
6. The method of claim 1 wherein the transformed plant is selected from the group consisting of sunflower, soybean, canola, alfalfa, corn, wheat, rice, and barley.
7. The method of claim 1 wherein the transgenic plant is a vegetable plant.
8. The method of claim 1 wherein the non-selectively assayable transgene is selected from the group consisting of an oxidase gene, a decarboxylase gene, a green fluorescent protein gene, the GUS gene, a luciferase gene, a Bt crystal protein toxin gene, and an anthocyanin gene.
9. The method of claim 1 wherein the plant tissue is assayed using a technique selected from the group consisting of PCR, an enzymatic assay, an ELISA, a bioassay, and a biochemical analytical process.
10. A transformed plant regenerated using the method of Claim 1, 2, 3, 4 or 5.

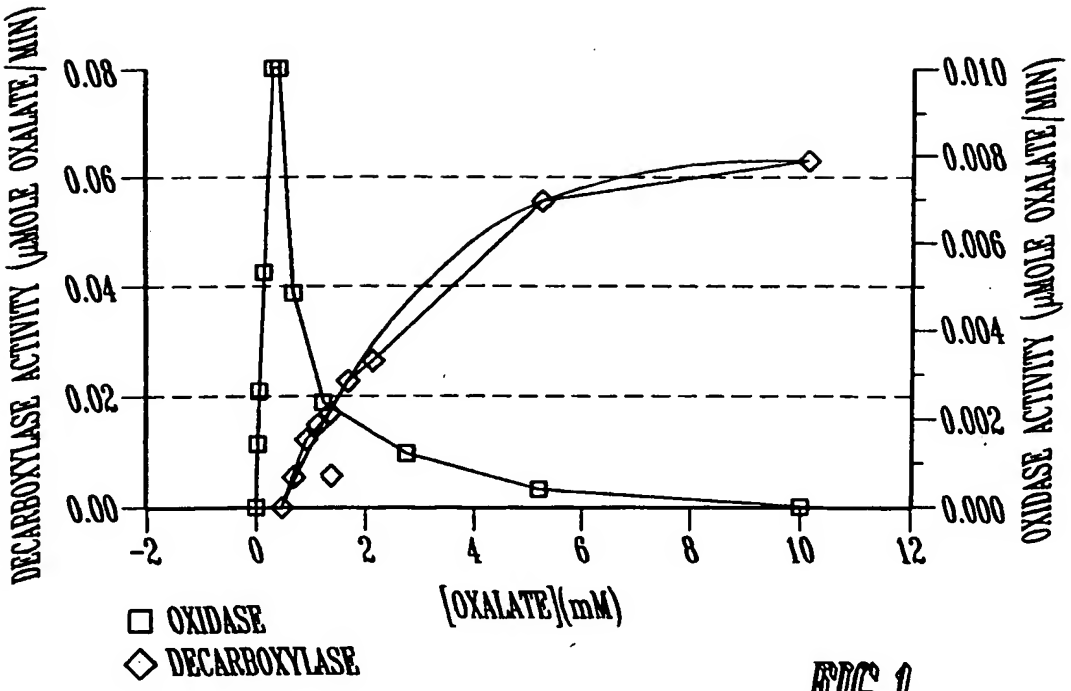
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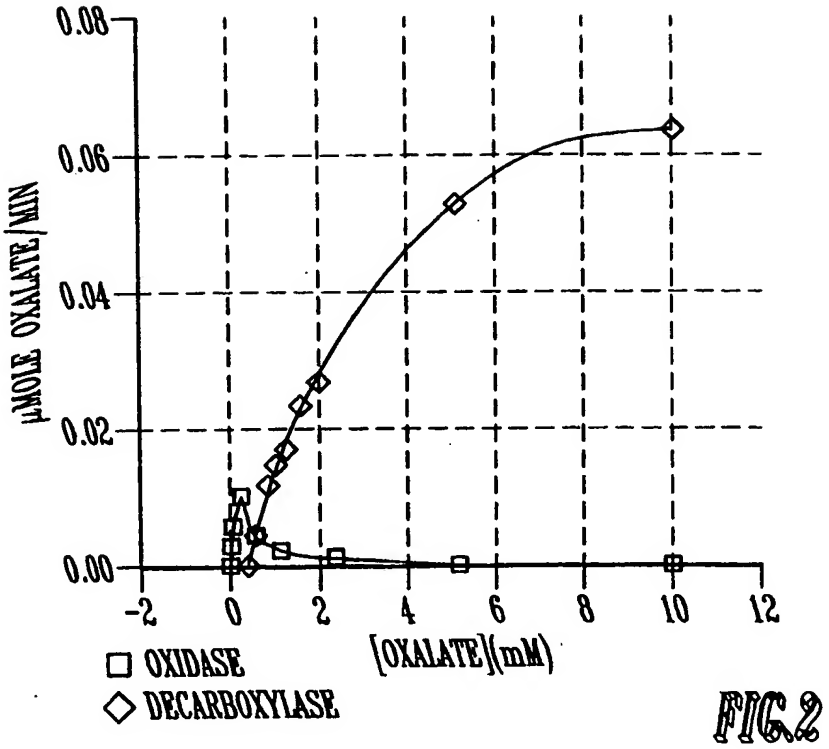
FIG 1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DECARBOXYLASE

FIG.2

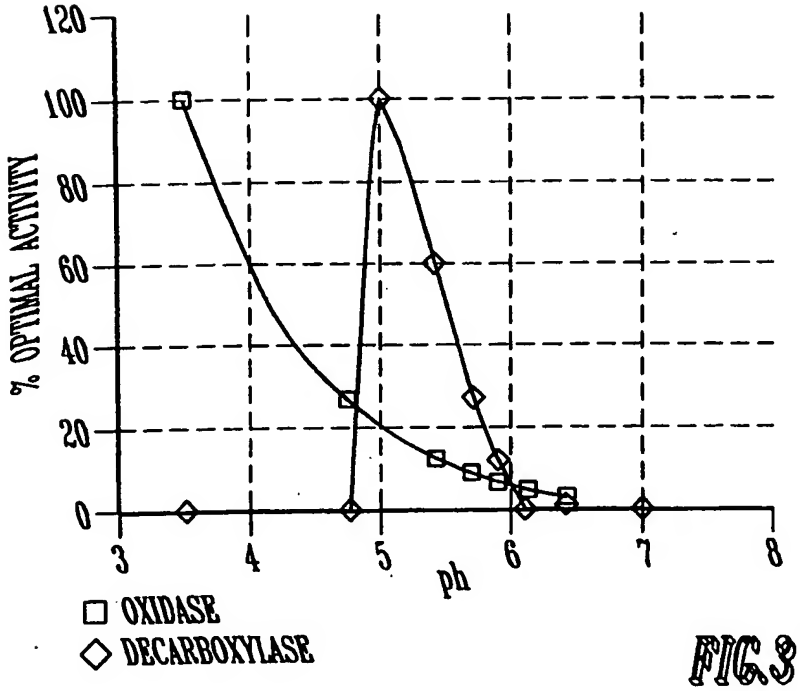
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FIG.3

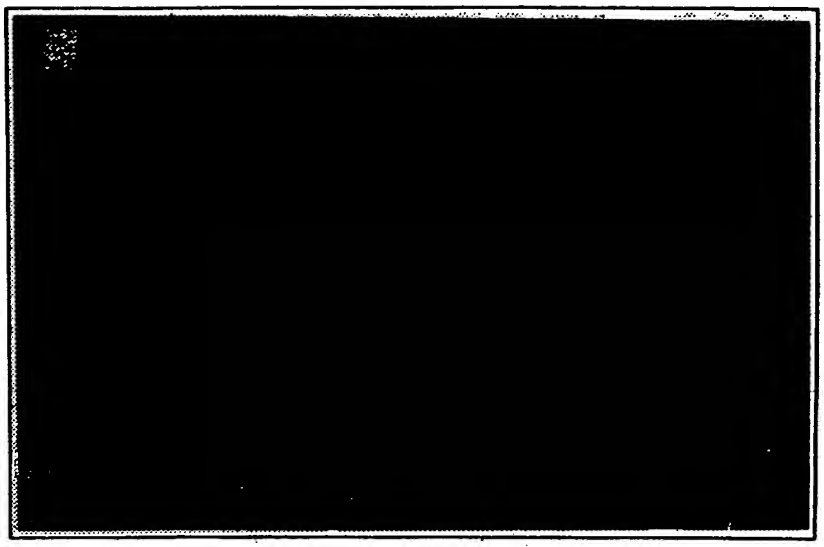

FIG. 4A

FIG. 4B

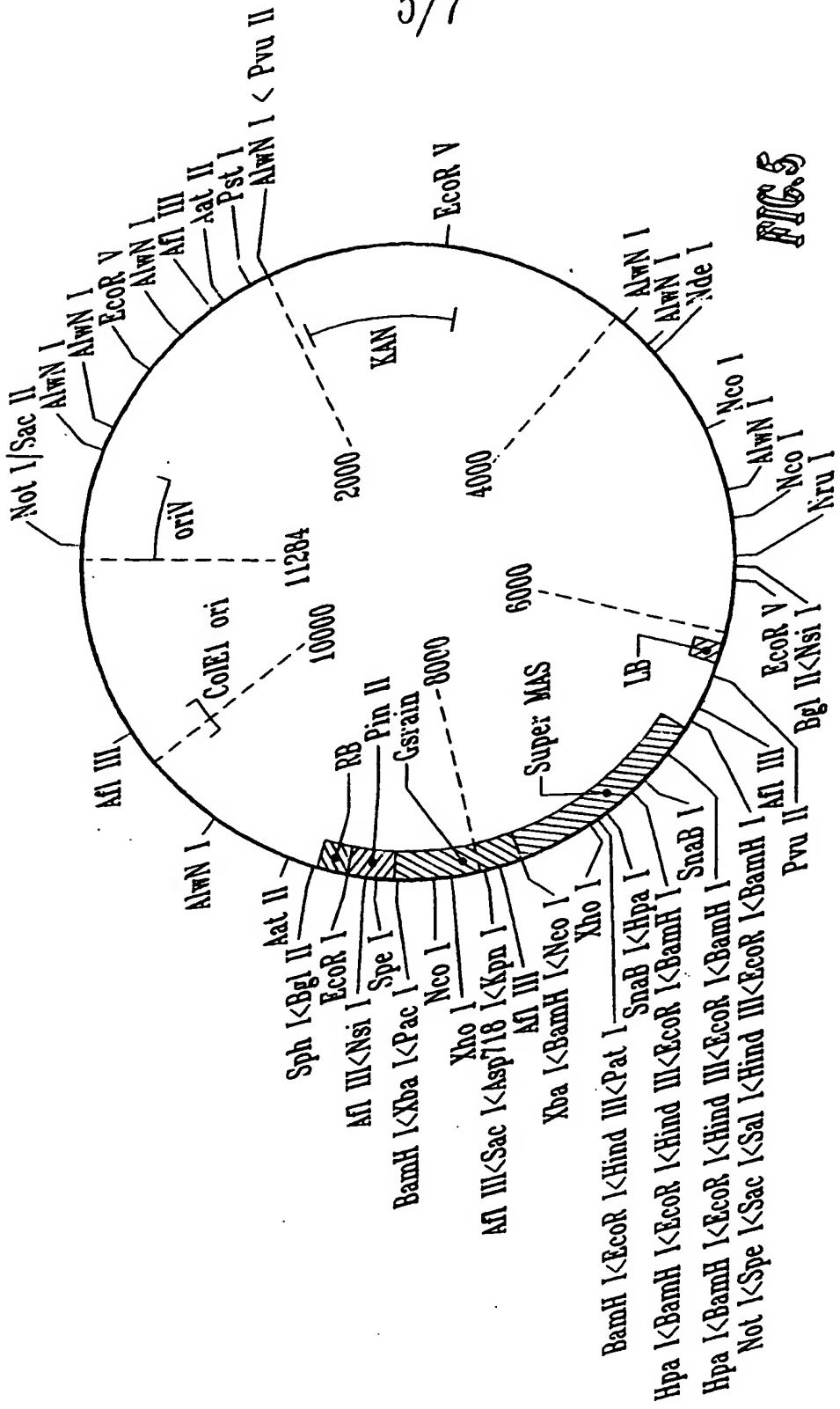
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FIG 5

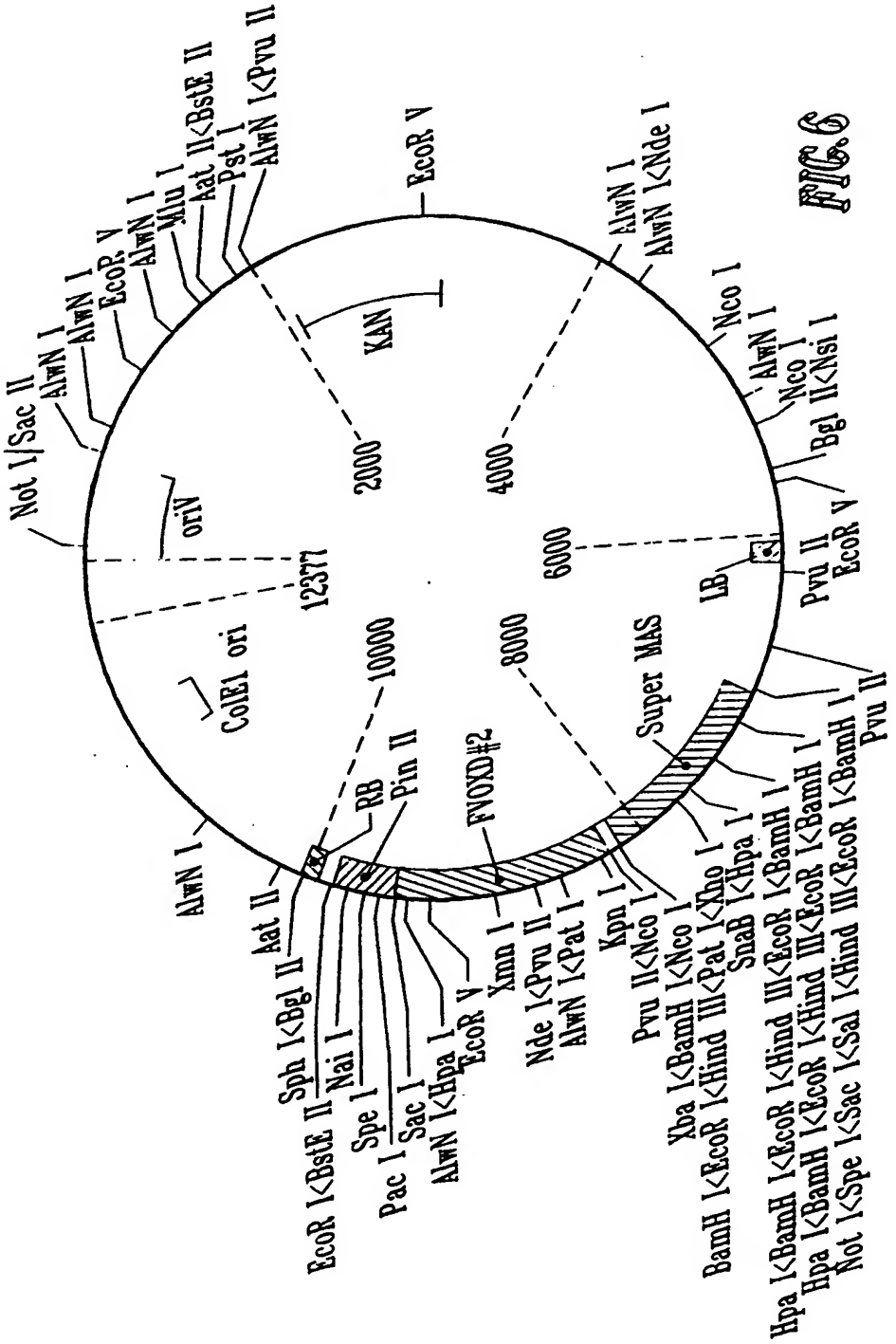
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FIG 6

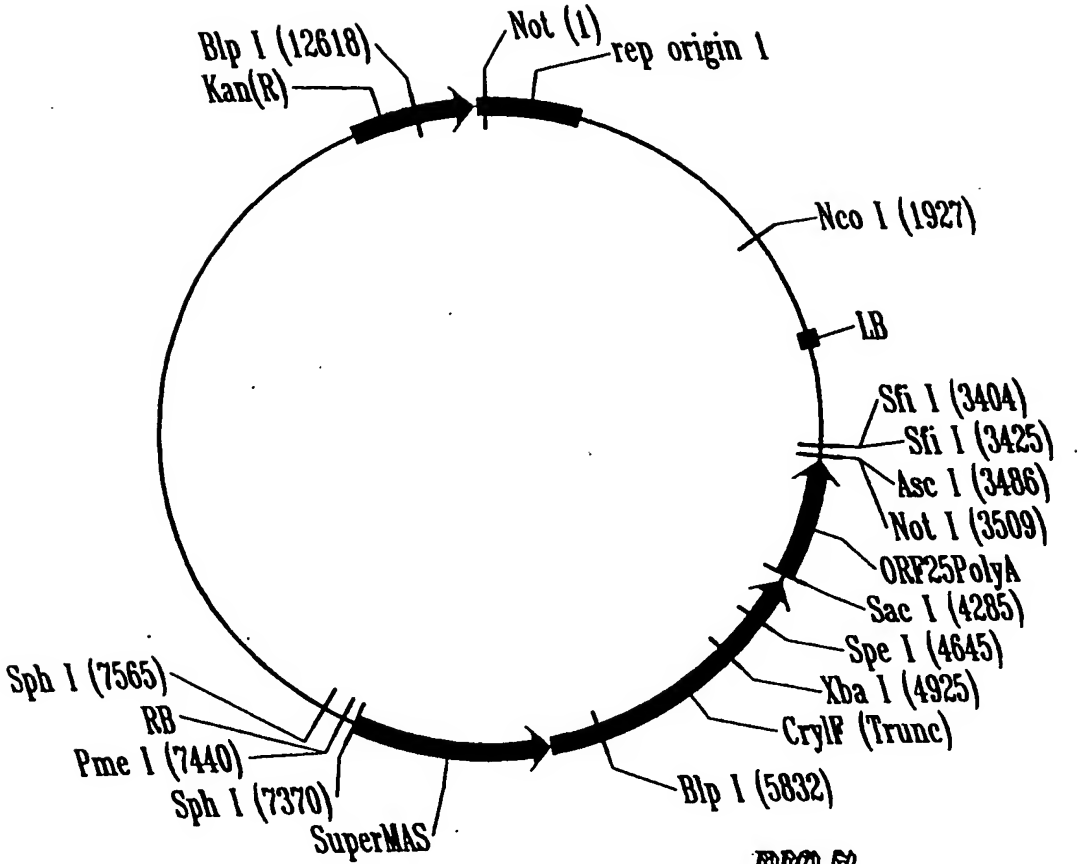
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FIG. 7